International Journal of Pharmaceutical Frontier Research

http://www.ijpfr.com

Preliminary phytochemical screening and *in vitro* antibacterial evaluation of the leaf and root extract of *Azadirachta indica* Plant.

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The plant Neem (*Azadirachta indica)* is used traditionally for treatment of various illnesses in Ethiopia. However, there was paucity of information with regard to antibacterial activity of part of Neem used. So in our study we evaluate the antibacterial activity of Neem plant. An experimental study design was conducted to evaluate *in vitro* antibacterial activity of the plants from January to May 2010. The n-hexane, methanol and acetone extracts of the leaves and roots of Neem were screened for their antibacterial activity using the Cup plate agar well diffusion method. They were tested against four bacteria; one Gram-positive bacterium (*Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi*). The susceptibility of the microorganisms to the extracts of the plant was compared with selected antibiotics. The antibacterial activities of Neem were discussed according to their phytochemical components. Most of the gradient extracts of Neem extracts of leaf and root shows dose dependant antibacterial activity. Among all the extracts tested, the acetone and methanol extract was found to be the most active against all the selected strains. The antibacterial activities of the extracts are probably because of polyphenolic compounds and alkaloids detected. Although, this study provides some evidence concerning the claimed biological activities of these plants, it is not adequate. So, further work should be done to show the potential of these plants for further development of modern therapeutic agents.

Key words:-Antibacterial activity, plant Neem gradient extracts.

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INTRODUCTION

The traditional medicinal plants have been used to prevent and treat various health problems. The majority of the population in developing countries still relies on herbal preparations to help enhance health. This is also true in Ethiopia where the great majority of the populations still rely on plants for their healing action in various health problems [1].

Ethiopians peoples have their own set of written or oral pharmacopoeias with medicinal use of some species being restricted to each ethnic group. An indigenous knowledge medicinal plant is uneven across the different zone/ locations with different religious, linguistic and cultural background of people. *Azadirachta Indica*, commonly referred to in many countries as the Neem tree, is a member of the *Meliaceae* family. This broad-leaved evergreen can reach heights of 30

meters with a trunk girth of 2.5 meters. It grows in the rift valley areas of Ethiopia where it is traditionally used as mosquito and fly repellent.

Traditional healings and remedies made from plants play important roles in the health care of millions of people in the developing and underdeveloped countries of the world. The Study is important to confirm the claimed antibacterial effect of the plant and their help to fill the gap in knowledge about the medicinal value of Neem extracts of leaf and root i.e. integration of traditional medicine and modern medicine.

The traditional medicine was contribute its own value in search of new chemotherapeutic agent in the world facing the problem of a resistant to synthetic agent and the toxicity of synthetic agent from most known novel chemotherapeutic agent. This study may help to provide a document on in vitro antibacterial activity of Neem extracts of leaf and root of Ethiopian origin and helps as base line information for further study on it.

Medicinal plants used as inspiration for new drug provides on infusion of novel compound or substances for healing disease. Evaluation plant from traditional African system of medicinal plants provides us with calves as to how these plants can be used in treatment of disease. In Ethiopia, although Neem tree is used for traditional treatment various diseases in Ethiopia, its anti microbial (bacterial) activity has not been evaluated so far hence the present work is intended to evaluate for antibacterial properties of the plant leaf & root extraction of Neem.

MATERIAL AND METHODS

Plant materials

The plant species used during this investigation was Neem and the local name of the plant also Neem. The plants were collected in the month of January, 2010. The Neem plant species were collected from Arsi Asela area, 100 kMs south east of the capital Addis Ababa, Ethiopia. The fresh leaves and roots of Neem were taken to Jimma University herbarium, at the department of biology and taxonomically authenticated by Ato Ketisa Hundera, Jimma University Herbarium, Department of biology, and the voucher specimen was deposited (MS01, 2010) as Neem .The collected leaves and roots of the plant was aired, dried in the shade and were ground with electrical grinder. The powdered plant materials were stored in air tight amber glass containers until used for extraction.

Chemicals and reagents

The following chemicals, solvents and control drugs were used during this work. Methanol (Abron chemicals , batch No AB /500/et/07), n-hexane (BDH chemicals Ltd, Poole, , lot No 29739 ,England), acetone (Lab Merck chemicals Ltd , batch No 020411, India),distilled and sterilized water, Ketoconazole (domina pharmaceutiacals,batch No Ke806GT03,India), gentamicine (Himedia lab pvt Ltd,lot No S.W 1140).

Media for antibacterial activity screening

The culture media was employed for antibacterial screening. Muller Hinton agar, MHA (lot No X4225E, oxoid, England) and Nutrient broth (DEFCO laboratories, USA). All the culture media was prepared and treated according to the specific manufactures guidelines.

Test Organisms

In this study the bacterial strains commonly implicated in causing infections disease: *staphylococcus aureus* (Gram positive), *Pseudomonas aeruginosa* (Gram negative), *Escherichia coli* (Gram negative), *salmonella typhi*, were used for in vitro antimicrobial activity. And the activity was tested in Jimma University microbiology laboratory.

The bacterial strains for the *in vitro* antibacterial tests in this study was staphylococcus aureus ATCC25925 (Gram positive), Escherichia coli ATCC25922 (Gram negative), salmonella typhi ATCC83859 (Gram negative) and pseudomonas aeruginosa ATCC27853 (Gram Negative). The organisms was obtained from the department of infectious diseases, Ethiopian health and Nutrition research institute, EHNRI, Addis Ababa, Ethiopia. The microorganisms were selected because they were of great public health importance. *S. aureus* is known to cause a wide range of illnesses, from minor skin infections (such as pimples, boils and cellulites) and abscess to life threatening diseases

such as pneumonia, meningitis, Endocarditic, toxic shock syndrome (TSS) and septicemia. *P. aeruginosa* is an important opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. It causes urinary tract and respiratory system infections, dermatitis, soft tissue infections, bone and joint infections, gastro intestinal infections and a variety of systemic infections, particularly in patients who was immuno compromised. *E.coli* and *S. typhi* are also known to cause a very serious infection.

Preparation of the gradient extracts

Powder plant part of Neem extracts of leaf (100gm) and root (80gm) was packed in a thimble and successive extract was under taken by sox let apparatus using n-hexane 500ml (60-80 0^c), acetone 500ml and 80% methanol (v/v) 500ml as a men strum until the last portion of the extract becomes colorless. Each fraction was collected separately and concentrated under the reduced pressure using a rotary evaporator at about 40 0^c and the semisolid mass was dried in an oven at about 40 0^c and the dried mass was then weighed and the percentage yield calculated.

The stock solutions of the dried mass from the gradient extracts were prepared by taking 1gram of the extract to make the final solution 10ml. In this way the solution with concentration of 100µg/µl was prepared Solution showing good activities during the pilot study was subjected to antibacterial activity testing using standard procedure.

Bioactivity testing of the extracts

The antibacterial activity of the gradient solvent extracts against the selected microorganisms was performed by the agar well disc diffusion method.

Preparation of inoculums

All microorganisms used as test organisms were derived from stock cultures of Department of Microbiology and transferred into a tube containing 5ml of nutrient broth medium and then vortexes thoroughly. The broth culture was incubated in an incubator at 37^0c for about an hour to reactivate the organisms. The optical density of actively growing broth culture measured spectrophotometrically at 625 nm and absorbance value adjusted between 0.08 to 0.1. The adjusted suspension represents a 1-2X10^7CFU of bacterial strains per ml [2].

Application of the extracts

Separate agar plates were prepared as follows for each organism. 20 ml sterile molten Muller Hinton agar was poured into sterilized Petri dish (10mm diameter) and set aside until solidified or congealed.

Following this, the inoculums suspension with adjusted turbidity was inoculated by using sterile cotton swab. During inoculation, a sterile cotton swab was dipped into the adjusted suspension by rotating several times and pressing firmly on the inside wall of the tube above the fluid level so as to remove excess inoculums from the swab. To ensure even distribution of inoculums, the plate was rotated approximately 60^0 each time and finally rim of the agar was swabbed [3].

The sock solution having 100mg/ml concentration was prepared by dissolving the dried mass of the gradient extract (1 gm) in the selected solvents to make 10ml and subjected to antibacterial activity tests at different concentrations. N-hexane, acetone and methanol extracts. The acetone extract was dissolved in acetone while the methanol extract was dissolved in the methanol itself and the n-hexane extract was dissolved in the n-hexane it self.

The dried mass of the gradient extracts (1 gram in 10ml solution) were prepared and different concentrations (4mg/ml, 2mg/ml, 1.5mg/ml,1mg/ml and 0.5mg) of the extracts from the stock solution having the concentration of 100µg/ml was taken and subjected to antibacterial activity tests. The n-hexane, acetone and methanol extract was dissolved by itself. Agar well diffusion discs was prepared by using micropipette tip having 6 mm diameter size. All the extracts of different amounts of (40µl, 20µl, 15µl, 10µl and 5µl) was prepared and inoculated into empty well of agar media discs. The extract of the plants was applied immediately on solid Agar medium by pressing gently. On the solid agar plate an impregnated into empty with only the solvent (acetone, methanol and n-hexane) and commercial discs of gentamicine (10µg, HIMEDIA) was used as negative and positive controls, respectively. The sterilized Petri dishes were then incubated at 37 0c for 24 hours.

At the end of the period, the antibacterial activities of gradient extracts were evaluated by measuring the clear zone of inhibition formed using vernier caliper in millimeter.

Determination of minimum inhibitory concentration

Determination of minimum inhibitory concentration of the crude extracts of Neem root and leaf gradient extraction was determination by agar diffusion methods. The Muller Hinton agar was first prepared in a way described by the manufacturer and then sterilized by autoclaving. The 18ml of the molten agar was mixed with 2ml of different concentration of the extracts and then, the mixture of the media and the extract was thorough mixed and poured into pre-labeled sterile Petri-dishes on a level surface. Additional Petri-dishes containing only the growth media was prepared in the same way so as to serve for comparison of growth of the organism, the plates were then set at room temperature until it solidified. The sufficient suspensions of the test organisms were inoculated onto the series of agar plates using standard loop. The plates were then be incubated at 37 0^c for 18-24 hours. The lowest concentration or highest dilution which inhibited the growth of the respective organisms was taken as MIC. Average data of triplicate analysis were reported.

Phytochemical screening

The method developed by Dawo (2001) was used to detect the presence or absence of the main secondary metabolites groups in the gradient solvent extracts of Neem extracts of leaf and root**.** All results of the phytochemical screening are presented in table 1.

TABLE 1 Result of the phytochemical screening of the extracts of Neem (*Azadirachta indica*) extracts of leaf and root. January to May 2010

Phytochemical constituents	Reagent	Fraction analyzed					
		Leaf n-hexane	Leaf Acetone	leaf 80% Methanol(v/v)	Root 80% Methanol(v/v)	Root n-hexane	Root Acetone
Alkaloid	Dragendorff;s -Meyer;s test	+	+	+	+	-	+
Carotinoid	-Antimony trichlorid	+	+	_	_	-	-
Saponins	-Honey comb froth test	+	+	+	+	-	+
Anthraquinone	-Borntrger;s test	+	+	_	_	-	-
Polyphenols	-1% FeCl3	_	+	+	+	-	+
Cardiac glycoside	-Keller kiliant -Lieberman Druchards	+	+	+	+	-	+
Flavonoids	-Ammonia vapour	_	+	_	_	-	-
Phenolic glycoside	-1% FeCl3 & -1% k3Fe(CN)6	_	+	+	-	-	-
Phytosterols	-3%vanillin inconc. H2SO4 -Sakowski test	+	+	_	+	-	+

KEY: (-) absent, (+) present

Based on the test results, phytosterols and carotenoids were detected in the n-hexane and acetone fractions of leaves of Neem extracts and polyphenolic compounds were detected in the methanol, acetone leaf fractional extract of the plant and also in root methanol and acetone extract. Phytochemical screening reveals the concentration of alkaloids, carotinoids, anthraquinones, polyphenols, and cardiac glycoside. In addition to this both n-hexane and acetone extraction has demonstrated carotinoids, anthraquinones and cardiac glycoside of bioactive components. But the n-hexane and methanol extraction of Neem root did not reveal bioactive components of anthraquinones, flavonids, carotinoid and phenolic glycoside.

RESULTS AND DISCUSSION

Antibacterial activities of the gradient extracts

The antibacterial activities of the gradient extracts of Neem leaf and root gradient extraction are summarized in the table-2.

TABLE 2 Antibacterial activities of the gradient extract of Neem (*Azadirachta indica*) root gradient extraction January to May 2010

Fraction analyzed	Concentration (µg)	Activity against pathogen			
		Ssa	Psa	Esc	S.typhi
N-hexane	50	-	-	-	-
	100	-	-	-	-
	150	-	-	-	-
	200	-	-	-	-
	400	-	-	-	-
Acetone	50	++	+	+	++
	100	++	+	++	++
	150	+++	++	+++	+++
	200	+++	+++	+++	+++
	400	+++	+++	+++	+++
Methanol	50	-	-	-	+
	100	+	+	-	++
	150	++	++	-	+++
	200	+++	+++	++	+++
	400	+++	+++	++	+++
Gentamicine	10µg	+++	+++	+++	+++

Ssa- *staphylococcus aureus* (Std), Psa – *pseudomonas arogenosa* (Std) , Esc- *Escherichia coli* (Std) , and S. typhi –Salmonella typhi (std)

The zone of inhibition of bacteria around the disc was measured and the assay was scored positive (+) if it was < 2mm, doubly positive (++) if the zone was 2-6mm, triple positive (+++) if the zone was ≥7mm and negative (-) if there was no inhibition of microbial growth. The microbial assay results were compared with commercial antibiotics i.e. discs containing gentamicine sulphate (10µg)

The n-hexane, acetone and methanol extracts of the leaf and root of Neem were subjected to a preliminary screening for antimicrobial activity against four standard bacteria (*Staphylococcus aureus*, *Escherichia coli*, ,*Pseudomonas aeruginosa* and *Salmonella typhi*) .It was clear from table 2 and 3, that both acetone and methanol of the leaf Neem and n-hexane, acetone and methanol root

extract of Neem showed some activity against some organisms tested but the root extract of n-hexane no activity against the four standard bacteria .

TABLE 3 Antibacterial activities of *Neem* (*Azadirachta indica*) root gradient extraction January to May 2010

Part of the Neem (*Azadirachta indica*) taken	Concentration (µg)	Zone of inhibition(mm)			
		Ssa	Psa	Esc	S.typhi
Root Methanol extract	50	-	-	-	2
	100	2	3	-	5
	150	3	5	-	8
	200	8	7	3	12
	400	12	13	4	15
Root acetone extract	50	4	2	2	3
	100	5	2	4	5
	150	9	5	9	7
	200	12	8	13	10
	400	18	11	16	19
Gentamicine	10µg	26	15	20	24

(-)No inhibition zone, Ssa- *staphylococcus aureu*s **(Std), Psa –** *pseudomonas arogenosa* **(Std) , Esc-** *Escherichia coli* **(Std)** and **S. typhi –**Salmonella typhi **(std)**

The acetone and methanol leaf extract no activity against Escherichia coli and salmonella typhi respectively and also methanol root extract are dose dependant activity against pseudomonas aeruginosa, and staphylococcus aureus.

The acetone leaf extracts of Neem antibacterial activity against four standard bacteria (*Staphylococcus aureus*, *Escherichia coli*, ,*Pseudomonas aeruginosa* and *Salmonella typhi*) and also Methanol and acetone extract of Neem showed activity against some of the four standard bacteria the effect are dose dependent but methanol extract of leaf no activity against salmonella typhi .

As shown in the table 4, some of the fractions (n-hexane, acetone and methanol) exhibited activity against *S. aureus* and *P. aeruginosa* in a dose dependant manner and also as shown in table 3 the acetone and methanol extract exhibited activity against *S. aureus* and *P. aeruginosa* in a dose dependant manner but the n-hexane extract of the root not activity against the four standard bacteria. The acetone and methanol extracts are also active against *E.coli* at high dose and this is also dose dependant activity.

Table 5, reports the antibacterial activities of the Neem leaf and root gradient extraction. Against one strains of gram positive and three gram negative bacteria species using agar well diffusion technique on solid media.

TABLE 4 Antibacterial activities of the gradient extract of Neem (*Azadirachta indica*) leaf gradient extraction January to May 2010

Fraction analyzed	Concentration (µg)	Activity against pathogen			
		Ssa	Psa	Esc	S.typhi
N-hexane	50	++	-	-	-
	100	++	++	-	-
	150	++	++	++	-
	200	++	++	++	-
	400	++	++	++	-
Acetone	50	+++	++	++	+++
	100	+++	++	++	+++
	150	+++	+++	+++	+++
	200	+++	+++	+++	+++
	400	+++	+++	+++	+++
Methanol	50	-	-	-	-
	100	-	-	++	++
	150	-	-	++	++
	200	++	++	+++	+++
	400	++	+++	+++	+++
Gentamicine	10µg	+++	+++	+++	+++

Ssa- *staphylococcus aureus* **(Std), Psa –** *pseudomonas arogenosa* **(Std) , Esc-** *Escherichia coli* **(Std)** and **S. typhi –** *Salmonella typhi* **(std)**

The methanol extract of leaf of Neem showed pronounced activity (4mm-15mm) against *Escherichia coli and* (4mm-15mm) against *Salmonella typhi* and less active against *Staphylococcus aureus(3mm-6mm),* and *pseudomonas aeruginosa(3mm-9mm).* and acetone extract of leaf showed pronounced activity against the four standard bacteria ,high activity against *Staphylococcus aureus* (8mm-20mm), *Escherichia coli* (5mm-18mm), ,*Pseudomonas aeruginosa* (3mm-12mm) and Salmonella typhi(7mm-17mm) .

The methanol extract of root Neem showed pronounced activity against the four standard bacteria *Staphylococcus aureus* (2mm-12mm), *Escherichia coli* (3mm-4mm), ,*Pseudomonas aeruginosa* (3mm-13mm) and *Salmonella typhi* (2mm-15mm). .Antibacterial activity observed in root extract of acetone Neem activity against the four standard bacteria *Staphylococcus aureus* (4mm-18mm), Salmonella typhi(3mm-19mm)

, *Pseudomonas aeruginosa* (2mm-11mm*)* and high activity against *Escherichia coli* (2mm-16mm).and also the n-hexane extract of root no effect.

The inhibitory effect of different concentration of Neem extract was examined by direct visual comparison of the test cultures with the control cultures. The minimum inhibitory concentration (MIC) recorded as the lowest concentration of Neem extract that was capable of completely inhibiting the growth of the test organisms (Table-5).

TABLE 5 Minimum inhibitor concentration (MIC) values of crude acetone extracts of root *Neem (Azadirachta indica) against standard strains* January *to May 2010*

Concentration (mg mL-1)	*S. aureus ATCC25923*	*P.aroginosa ATCC27853*	*S. typhi ATCC83859*	*E. coli ATCC25922*
1gm/ml	+	+	+	+
0.75gm/ml	+	+	+	+
0.5gm/ml	-	-	+	+
0.25gm/ml	-	-	-	-
0.01gm/ml	-	-	-	-

N.B: (+) indicates inhibition of bacterial growth, (-) indicates bacterial growth

We found out that the use of these medicinal plants as traditional remedies for treatment of some infections and also the potential of these plants for the development of modern antimicrobial agents is justifiable. Most of the plant extract showed various inhibitory effects against all the micro organisms tested except the n-hexane root which was found to be ineffective against the four standard bacteria. The acetone leaf extracts of Neem, antibacterial activity against four standard bacteria (Staphylococcus *aureus*, *Escherichia coli*, Pseudomonas *aeruginosa* and *Salmonella typhi)* and also Methanol extract of Neem showed activity against four standard bacteria but the effect are dose dependent.

The yield (on dry weight basis) of the gradient extracts of Neem extracts of leaf i.e. n-hexane, acetone and methanol extract was found to be 3.5gm(3.5%), 4.5gm(4.5%) and 11.91gm(11.91%) respectively and Neem extracts of root i.e. n-hexane, acetone and methanol extract was found to be 1.5gm(1.875%), 2gm(2.5%) and 3.5gm(4.375%), respectively. The yield from the methanol fraction was found to be the highest as compared to the other two fractions. Such a high yield could be an indication for the extracting power of the solvent with respect to semi polar and polar components. It means also, if the fraction is found to be active and promising for further development, can add an advantage to the commercial production of these plants. The effectiveness of the methanol and acetone extract of this plant, as observed in this study, could be an indication for further use of this herbal drug for the treatment of various infectious diseases.

In this work most of the extracts revealed dose dependant activity in one or more of the tested organisms. The probable reason for this activity is found to be the major secondary metabolites detected to be present like alkaloids, flavonoids, and diterpenoids.

Of all the different extract tested, the methanol extract and acetone both root and leaf extract had broad spectrum of activity followed by n-hexane extract, respectively. This suggests that the antibacterial active principles are most probably polar in nature [4]. It had antibacterial activity at all test concentrations against *S. aureus* and P. *aeruginosa* except at concentrations less than 100µg. It is also believed that plants that are rich in a wide variety of secondary metabolites belonging to the different chemical class's terpenoids, alkaloids, and polyphenolic compounds like flavonoids exhibit various biological activities including antibacterial activities[5] . Hammond reported that this plant chemically include alkaloids ,cyanogenic glycosides , saponins , cardiac glycosides ,

tannins and simple phenol compounds , due to the content of these secondary metabolite it have good antibacterial activity the extract of leaf and root extract of Neem plants[6] .

The study done in other country shows all extracts of root and leaf Neem plant extract were inactive against *Escherichia coli* but in our study showed pronounced effect methanol and acetone extract of root and leaf Neem active against Escherichia coli but the effect is dose dependent . The methanol extract of Neem exhibited pronounced activity against *Bacillus subtilis* (28 mm), high activity against the Gram-positive organism *Staphylococcus aureus* (18mm), the Gram-negative bacteria *Proteus vulgaris* (18 mm) and *Salmonella typhi* (20 mm), low activity against *Pseudomonas aeruginosa* (14 mm) and acetone of leaf inactive against *Escherichia coli* .

Both extracts from the leaf and the root were effective in inhibiting the growth of bacteria. The methanol and acetone extracts showed pronounced activities against most of the bacteria. The presence of these compounds may explain the antibacterial activity of these plants as compared that of the n- hexane extracts of the Neem plants.

The methanol extract of Neem exhibited pronounced activity against *Bacillus subtilis* (28 mm), high activity against the Gram-positive organism *Staphylococcus aureus* (18mm), the Gram-negative bacteria *Proteus vulgaris* (18 mm) and *Salmonella typhi* (20 mm), low activity against *Pseudomonas aeruginosa* (14 mm) and inactive against *Escherichia coli* . These might be due to presence of triterpenoids, phenolic compounds, Carotenoids, steroids, valavinoids, ketones and tetratriterpenoids azadirachtin these results were similar to those reported by Ikram and Inamul 1980a, 1980b**.** All extracts were inactive against *Aspergillus niger*

In general, the antibacterial activity of *Neem* are weak compared to the standard positive controls used: gentamicine which showed universal activity against all bacterial strains with inhibition zone ranging from 15 - 26mm and the Neem extract with inhibition zone of 2 - 20mm against some bacterial strains This indicates that, these extract had high potential antibacterial effect and promising for further development of modern drugs.

CONCLUSION

Overall, the results support partly the use of these medicinal plants as traditional remedies for treatment of some infections and also the potential of these plants for the development of modern antimicrobial agents. Most of the plant extract showed various inhibitory effects against some the micro organisms tested except the n-hexane root extract which was found to be ineffective against the four standard bacteria and the methanol extract of leaf less active against *staphylococcus aureu*s. The gradient extracts of leaf and root Neem also show dose dependant activity against the tested organisms.

The antibacterial activities of Neem extracts may be due to polyphenolic compounds (flavonoids), alkaloids and higher terpenoids which are detected to be present in the plant.

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